Research article

Delayed union of femoral fractures in older rats:decreased gene expression

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Abstract

Background: Fracture healing slows with age. While 6-week-old rats regain normal bone biomechanics at 4 weeks after fracture, one-year-old rats require more than 26 weeks. The possible role of altered mRNA gene expression in this delayed union was studied. Closed mid-shaft femoral fractures were induced followed by euthanasia at 0 time (unfractured) or at 1, 2, 4 or 6 weeks after fracture in 6-week-old and 12-15-month-old Sprague-Dawley female rats. mRNA levels were measured for osteocalcin, type I collagen α1, type II collagen, bone morphogenetic protein (BMP)-2, BMP-4 and the type IA BMP receptor.

Results: For all of the genes studied, the mRNA levels increased in both age groups to a peak at one to two weeks after fracture. All gene expression levels decreased to very low or undetectable levels at four and six weeks after fracture for both age groups. At four weeks after fracture, the younger rats were healed radiographically, but not the older rats.

Conclusions: (1) All genes studied were up-regulated by fracture in both age groups. Thus, the failure of the older rats to heal promptly was not due to the lack of expression of any of the studied genes. (2) The return of the mRNA gene expression to baseline values in the older rats prior to healing may contribute to their delayed union. (3) No genes were overly up-regulated in the older rats. The slower healing response of the older rats did not stimulate a negative-feedback increase in the mRNA expression of stimulatory cytokines.

Background

While the radiographic and histologic progression of fracture healing is well understood, there are less data on the cytokines that regulate and control the healing process at the cellular level. The bone morphogenetic proteins (BMP) have been found to be expressed in the fracture callus by immunostaining of the protein [1,2,3] as well as by measurement of mRNA [4,5] and *in situ* hybridization [6,7]. The receptors for BMP are also up-regulated during fracture repair [1,2,8]. Morone *et al.* [9] observed increased mRNA expression of both matrix genes and BMP genes during the healing of spinal fusions in rabbits. There was a sequential expression of these genes in that BMP-6 peaked first, followed by BMP-4, then BMP-2, and last by a second peak of BMP-6 expression [9]. The data for BMP expression during fracture repair, combined with the efficacy of the BMPs in stimulating bone induction and fracture healing, have

led to the concept that the BMPs are key molecules in the initiation of this healing process [10].

Derangement of the expression of BMPs and other skeletally active cytokines may be important in understanding the failure of healing in delayed unions. It is not clear why, in some fractures, the healing process seems to come to a halt and does not progress further without surgical intervention.

We have found that younger rats heal mid-shaft femoral fractures faster than do older rats [11]. In these earlier studies, one-year-old rats failed to achieve normal biomechanical strength within 24 weeks after mid-shaft femoral fracture [11]. In contrast, young rats achieved normal biomechanical strength by four weeks after fracture [12]. In this model, as the rats get older, it takes progressively longer for them to begin a periosteal reaction after fracture, and it takes longer to achieve bridging callus. This slowing of fracture healing with age has been reported in humans [13] as well as in rats [14,15]. In humans, the time to union increased with age for humeral fractures [13].

The reason for the slowing of fracture healing in older animals is not fully understood. For some time it has been known that there are changes in the periosteal cell layer with age [16]. Following fracture there is an increase in the rate of mitosis in the periosteum near the fracture site. This rate slows with age: Fewer cells enter mitosis, and more time is required for the cells to undergo mitosis [16]. This may reflect a reduction in the number of osteogenic stem cells available for skeletal repair in older individuals [17].

Alternatively, changes in the expression of the cytokines controlling fracture healing would also affect the rate of healing. Recent work has shown that the genes responsible for embryonic induction of skeletal tissue are also involved in fracture healing. The BMPs are prominently expressed during embryonic tissue induction (reviewed by [18]). BMP-2 and BMP-4 have been reported in the fracture callus of rats [19] and mice [20].

This led to the hypothesis that the delayed fracture healing in older individuals may be related to abnormalities in the BMP-signaling pathway. To test this hypothesis, bone fracture was induced in young and older rats, and gene expression was measured in the fracture callus at various times after fracture induction. Expression of osteocalcin, type I collagen, and type II collagen were measured as markers of fracture callus formation. BMP-2, BMP-4 and the type IA BMP receptor were measured as potential regulators of the healing process [2,9,19,20].

Materials and Methods

Rats

Intact female Sprague-Dawley rats were used at 6 weeks of age (136 ± 3 g body weight at fracture) or at 12-15 months of age (309 ± 6 g at fracture). They were purchased at 1 or 6 months of age respectively and housed with Teklad Rodent Diet (W) #8604 (Harlan, Madison, WI) and tap water *ad libitum*. This work was done in an AAALAC accredited vivarium under a protocol approved by the Institutional Animal Care and Use Committee.

Surgery

The animals were anesthetized with ketamine/xylazine at approximately 30 mg/5 mg per kg body weight. A small skin incision was made in the knee and the patella deflected. A hole was drilled into the intercondylar notch, and a stainless steel wire, 1.0 mm diameter (type 304 V, #O-SWGX-400, Small Parts, Inc., Miami Lakes, FL), was inserted into the medullary canal and recessed slightly. The muscle was sutured, and the skin closed with wound clips. As described earlier [11], a closed mid-diaphyseal fracture was induced using a Bonnarens and Einhorn fracture induction device [21]. The device was preset to 1 mm of travel. The 544 g weight was dropped from a height of 18 cm. for the young rats or 34 cm. for the older rats. Randomly selected intact rats of the same age were used for 0-time controls. Femoral radiographs were made at fracture, at one week after fracture, and at euthanasia.

Rats were euthanized at 1, 2, 4, and 6 weeks after fracture. The times were chosen based on the study by Morone *et al.* [9] and on our experience with fracture healing in younger rats [12]. The femora were rapidly harvested, and a portion of the femur was collected using a Dremel tool (Dremel, Racine, WI) with an ultra thin-flex diamond disc saw (Stoelting, Wood Dale, IL). Two cuts were made at right angles to the long axis of the femur on either side of the visible callus on the periosteal surface of the femur. The tissue collection included any portion of the femur whose external surface was altered by the fracture repair process. In practice, this collected bone sample was one-third of the overall femoral length, was centered on the callus, and included the callus, cortical bone, and marrow elements. The sample was frozen in liquid nitrogen and stored at -80°C until analyzed.

Gene Expression

Tissues were homogenized with a Polytron sonicating homogenizer (PT10/35, Brinkman Instrument Inc, Westbury, NY), and RNA was extracted with TRIzol (Gibco BRL, Gaithersburg, MD). The RNA samples (0.8 μg) were then reverse transcribed at 42°C for 15 minutes using random hexamers as primers. This was then amplified by 35 to 50 cycles of the polymerase chain reac-

tion (PCR, GeneAmp RNA PCR kit, N808-0143, Perkin Elmer, Foster City, CA), with primers (Table 1) designed for osteocalcin, type I collagen α1, type II collagen, BMP-2, BMP-4, type IA BMP receptor, 18S ribosomal RNA, or G3PDH (Cat. #5507, Clontech Laboratories, Inc, Palo Alto, CA). The cycling conditions were 95°C for 2 minutes; 80°C for 5 minutes (with hot-start addition of the Taq DNA polymerase); 35 to 50 cycles of 95°C for 15 sec and 58-62°C (annealing temp., Table 1) for 30 sec; and finishing with a single step of 72°C for 7 minutes. The resulting cDNA samples were separated by electrophoresis on a 1.5% agarose gel with ethidium bromide in 0.5X TBE buffer at 100 volts. A water blank lane and a lane containing 1.0 μg of a 100 bp DNA ladder (Cat. #15628-109, Gibco BRL) as a molecular weight marker were run on each gel.

The agarose gels were blotted to nylon membranes (Hybond-N+, Amersham, Arlington Heights, IL). The amplimers were UV cross-linked and hybridized with Rapid-Hyb buffer (Amersham) at 42°C using γ-^{32}P ATP end-labeled oligonucleotides (Table 1, G3PDH probe from Clontech) as probes. The membranes were washed with 6X SSC, 0.1%SDS; 4X SSC, 0.1%SDS; and 2X SSC, 0.1% SDS for consecutive 15 minute washes at 25°C. The radioactivity on the Southern blots was quantified with a Fujifilm Bio-Imaging Analyzer BAS-1500 with MacBAS Ver.2.5 software. Rectangles of constant area were defined and radioactivity was recorded in units of photo stimulated luminescence (PSL).

The samples were organized so that one sample from each time point for both ages was present on each gel and on each resulting blot. This resulted in a total of three blots for each gene. These three blots were hybridized at the same time with a single preparation of labeled probe.

Statistics

Individual treatment groups are presented as mean ± SEM. Comparisons were made with the Mann-Whitney U test. For the measurement of gene expression, there were three to four samples per time point per age except for the older rats at 2 (n = 2) and 6 (n = 1) weeks after fracture due to unanticipated attrition. For the younger rats at 0, 1, 2, 4, and 6 weeks after fracture there were 3, 3, 4, 3, and 4 rats per group respectively, while for the older rats there were 3, 3, 2, 3, and 1 rats per group respectively. The experiment was designed to have three rats per group. However, one older rat at 2 weeks after fracture was unusable due to bone infection. For the four rats designated for study at 6 weeks after fracture, two were lost due to the intramedullary pin backing out, necessitating early euthanasia, and one was infected and therefore unusable.

Table 1: Primer pairs and oligonucleotide probes used in this study.

Gene	Amplimer Size (bp)	Annealing T Temp. (C)	Sense	Antisense	Probe	Accession
Osteocalcin	411	60	TCTCTCTGCTCACTCTGCTG	ATTTTGGAGCAGCTGTGCCG	AGGTGGTGAATAGACTCCGG	M23637
Type I Collagen	410	62	AGGGACACAGAGGTTTCAGTG	ACCATTGGCACCTTTAGCACC	ATCGTTACCACGAGCACCAG	Z78279
Type II Collagen	563	62	TCATAGGACCTAAAGGACCTCC	TTCTCCCTTAGCACCATCGAG	AACTTTGCAGCCCAGATGGCT	L48440
BMP-2	570	60	ACGTCCTCAGCGAGTTTGAG	CACCTGGCTTCTCCTCTAAG	CTTCCCGACGCTTCTTCTTC	Z25868
BMP-4	601	60	ACTTCTGCAGATGTTTGGGCT	AGGTAACGATCGGCTAATCCT	CTATTTCGGGAGCAGGTGGA	Z22607
BMP-receptor	472	58	TTGTCGCCATGATCGTCTTC	AGGAAGTCATAGAGAGACCC	TAAAGGCCGGTATGGAGAAG	D38082
18S rRNA	321	66	AGGCCCTGTAATTGGAATGAGTC	TTATTCCTAGCTGCGGTATCCAG	AGCTCGTAGTTGGATCTTGG	K01593

Abbreviations: Base pairs (bp); temperature (Temp.). Accession refers to the entry in the Gen Bank database from which the primer pairs and probe were derived.

Results

Radiology

Radiographs were made of all rats after surgery, one week later and at the time of euthanasia. There were similar mid-diaphyseal fractures in both the younger and the older rats. At one week after fracture there was an obvious periosteal reaction in the younger rats. By six weeks after fracture the younger rats had healed their fractures with abundant mineralized callus, and remodeling was underway.

In contrast, there is little change in the radiographs of the older rats at one week after fracture. At six weeks after fracture, these older rats had not healed their fractures and showed only minimal periosteal reaction. All younger rats achieved bridging callus by six weeks after fracture, whereas none of the older rats achieved bridging callus by this time (data not shown).

Time course for gene expression

Most genes had the same pattern of low levels of expression in unfractured bone. There was an increased mRNA level after fracture, which led to a peak in activity at one or two weeks after fracture. Thereafter mRNA expression levels fell to very low levels at four weeks after fracture and undetectable levels at six weeks after fracture (Figs. 1 and 2). The 7 younger rats were well healed radiographically by this time, and their decreased gene expression was understandable.

However, for the 15-month-old rats reported in detail here, there was the same pattern of low level of gene expression at four to six weeks after fracture. Since these rats had not healed radiographically, the reduction in gene expression to baseline was more difficult to explain. Additional studies were done with 16 one-year-old rats, which confirmed this pattern. Seven of these one-year-old rats were studied at four weeks after fracture, and the other 9 one-year-old rats were studied at six weeks after fracture. These one-year-old rats had the same pattern of low-to-undetectable gene expression at four to six weeks after fracture. Thus, a total of 20 rats at 12-15 months of age have been studied at four to six weeks after fracture, and all had low-to-undetectable gene expression for both skeletal matrix genes and for BMP-related genes.

Matrix genes

Three genes were measured as an index of matrix synthesis: osteocalcin and type I collagen for bone and type II collagen for cartilage. Both osteocalcin mRNA levels (Fig. 1, top) and type I collagen mRNA levels (Fig. 1, middle) tended to be higher in the unfractured (0-time) younger rats than in the 0-time older rats. Fracture resulted in a rise in gene expression in both age groups to a peak at two weeks after fracture. Thereafter, gene ex-

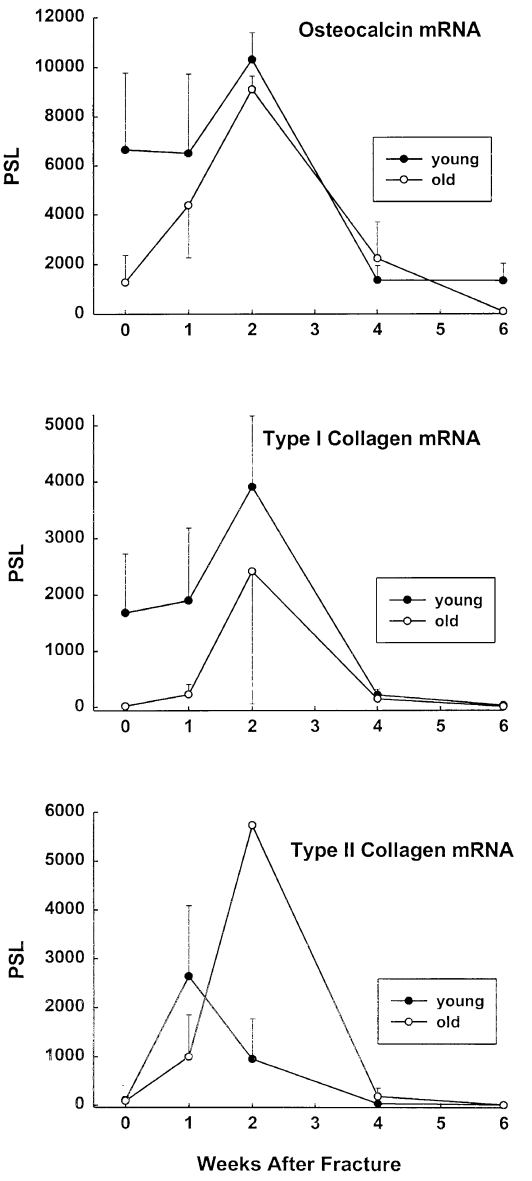

Figure 1
Change in the mRNA expression of osteocalcin (top), type I collagen α1 (middle), and type II collagen (bottom) with time after mid-diaphyseal femoral fracture in rats at 6 weeks (younger) and 15 months (older) of age. The mRNA was extracted, reverse transcribed, and amplified by the polymerase chain reaction. The amplimers were separated by electrophoresis, blotted to nylon membranes and hybridized with ^{32}P-labeled internal oligonucleotides. The level of radioactivity of each sample was measured in units of photo stimulated luminescence (PSL). The data are shown as mean ± SEM for three to four samples per point except for 2 (n = 2) and 6 (n = 1) week older rats. SEM smaller than the radius of the point are not shown.

pression levels fell to low levels, lower than the 0-time controls for each age group at four and six weeks after fracture (Fig. 1).

Type II collagen mRNA expression differed from that of type I collagen in being undetectable in the diaphyseal bone of the 0-time controls in both age groups (Fig. 1, bottom). This reflected a lack of cartilage synthesis in the intact diaphysis of the femur. Fracture resulted in a rise in gene expression for both age groups to a peak at one to two weeks after fracture. Both age groups decreased the mRNA expression of this gene at four and six weeks after fracture to undetectable levels (Fig. 1, bottom).

BMP-related genes

Three genes were measured to evaluate the regulatory control process over fracture healing. BMP-2 mRNA levels were undetectable in the 0-time controls of both age groups (Fig. 2, top). Fracture induced the expression of this gene. Expression peaked at one to two weeks after fracture for both age groups. BMP-2 expression for both age groups fell to undetectable levels at four and six weeks after fracture (Fig 2, top).

BMP-4 mRNA levels were also undetectable in the 0-time controls for both age groups (Fig. 2, middle). Fracture increased gene expression to a peak at one week after fracture in both age groups. There was a tendency for the older rats to have higher BMP-4 levels at weeks 1 and 2 after fracture ($P = 0.10$). Gene expression levels declined to undetectable levels at four and six weeks after fracture (Fig. 2, middle).

In contrast, the type IA BMP receptor mRNA levels were detectable in the 0-time control younger rats, but not in the older rats (Fig. 2, bottom). Fracture enhanced gene expression several-fold in both age groups to a peak at one week after fracture. The mRNA levels at four and six weeks after fracture were very low to undetectable in both groups (Fig. 2, bottom).

Housekeeping genes

Gene expression data are usually normalized to the expression of another gene whose mRNA levels are not affected by the experimental variables being studied. This controls for and corrects for variation in extraction, reverse transcription and the polymerase chain reaction. In this project, 18S ribosomal RNA was found to give a signal at each time after fracture in each sample. However, the 18S rRNA amplimer varied with time after fracture (data not shown).

Other housekeeping genes that are widely used were also studied and were found to vary in expression with time after fracture. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH), β-actin, and α-tubulin increased in both age groups following fracture. Expression then decreased in both young and older rats at four and six

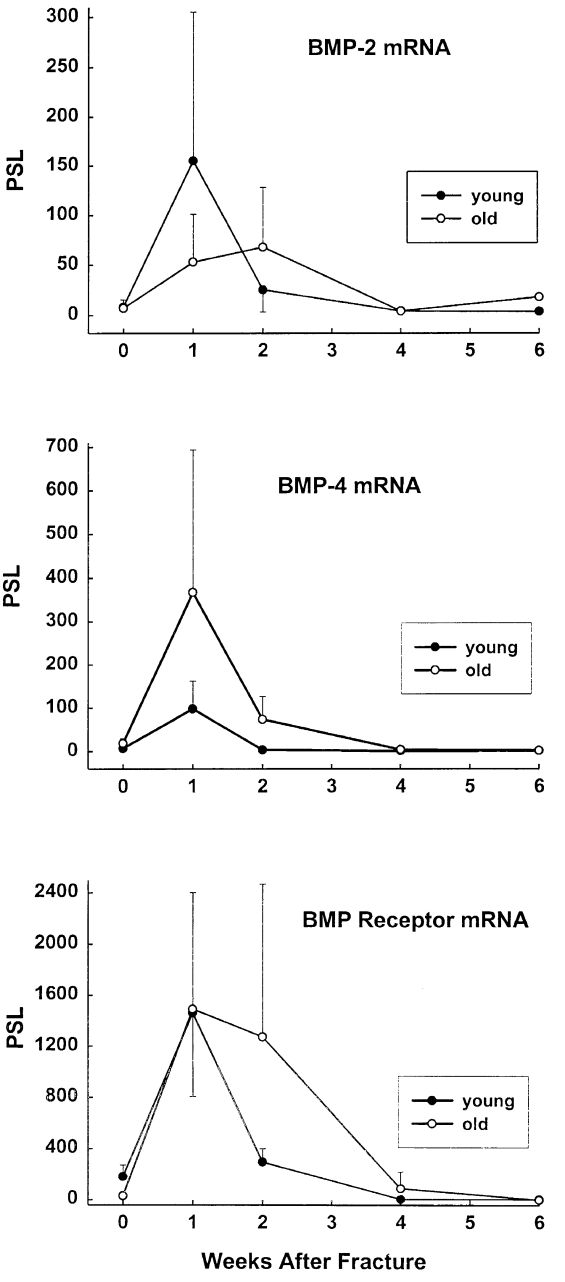

Figure 2
Change in the mRNA expression of BMP-2 (top), BMP-4 (middle), and the type IA BMP receptor (bottom) with time after mid-diaphyseal femoral fracture in rats at 6 weeks (younger) and 15 months (older) of age. The data are presented as in Fig. 1.

weeks after fracture (data not shown). This response to treatment made these genes unsuitable for normalization of the expression of the skeletally related genes that were described above.

Discussion

In this study we have measured mRNA gene expression in female rats for six weeks after femoral fracture. Females were chosen because female rats do not grow as large as male rats [22]. This then minimized the difference in body size between the two ages. Young mammals rapidly progress to fracture healing as we [11] and others [13,14,15] have shown before. In contrast, older rats and older humans heal more slowly [11,13,14,15]. In our earlier studies, comparable rats at one year of age failed to regain normal skeletal biomechanics within six months of femoral fracture, while younger rats regained normal biomechanics within four weeks after fracture [11]. In the present study the goal was to examine possible alterations in expression of genes related to the BMPs in order to explain this failure of the older rats to heal promptly. Despite the difference in age, all genes studied increased in expression after fracture followed by a decrease to baseline values by 4 weeks after fracture.

This decrease in the mRNA gene expression to undetectable levels at 4-6 weeks after fracture was the major conclusion of this study. The decrease occurred in rats of both ages and was independent of the healing status of the fractures. This was a surprising result, and occurred for both matrix and regulatory genes. It was unexpected since the genes under study were genes needed for fracture healing. We had anticipated that the older rats would have an up-regulation of regulatory gene expression to attempt to accelerate the healing process. We speculate that this decrease in gene expression is responsible for the failure of healing to progress more quickly in the older rats.

Our results appear to violate the principle of negative feedback control over biological processes. A slower healing sequence in older individuals might be caused by a diminished number of cells available to effect the repair [16,17]. This inadequate cellular response should lead to enhanced cytokine signaling by the BMPs to attempt to elicit a healing response by the bone cells. The enhanced signal should persist until healing is complete. The failure of enhanced BMP expression at four and six weeks after fracture in the older rats is difficult to explain. It may suggest that there is a constant time of stimulation of cytokine production following injury, rather than negative feedback control over the process of fracture healing. Injury may initiate a stimulus for bone healing that leads to enhanced BMP production for only a short time. For the younger rats, this time period seems adequate to allow healing to occur. For the older rats, a more prolonged signal may be required. The evaluation of this hypothesis will require additional experimentation.

There are changes in the periosteal cell layer with advancing age [16]. Following fracture there is an increase in the rate of mitosis in the periosteum near the fracture site. This rate is altered with age: Fewer cells enter mitosis, and more time is required for the cells to undergo mitosis [16]. This may reflect a reduction in the number of osteogenic stem cells available for skeletal repair in older individuals [17]. Despite this, we have extracted a similar amount of mRNA from the fracture site, with a similar increase in gene expression, in both the older and the younger rats. There was no evidence for a failure of gene expression for any of the genes studied thus far in the fracture callus of older rats.

The role of the BMPs has been studied in fracture healing in rats [2,3,5], mice [4,7,8], and rabbits [6] and in healing of spinal fusions in rabbits [9]. For the most part, these studies were done in younger animals who heal their bone fractures quickly. The rise in expression of the BMPs in these animals persists for the entire length of fracture healing through the point of formation of bridging callus. By correlating the gene up-regulation to the stage of fracture healing, it gives the impression that the BMPs are regulating all stages of fracture healing. In contrast, we have used age as an experimental model to slow the process of fracture healing [11]. This serves to separate the various radiographic stages of fracture healing and allows us to associate progression of healing with specific sets of up-regulated genes. In the present study, expression of BMP-2 and BMP-4, along with the type IA BMP receptor, decreased to baseline prior to the formation of bridging callus. This suggests that there may be a constant time of up-regulation of gene expression following fracture in the rat. Fracture is followed by four weeks of up-regulation of the stimulatory cytokines after fracture, and healing must take place within that window of time if it is to occur. The older rats cannot respond that quickly and fail to complete the healing process. Alternatively, these cytokines may only be needed for the formation of the soft callus. Once the soft callus is formed, these genes regress to baseline. Other genes, not yet identified, may be needed to stimulate the formation of bridging callus on the scaffold of cartilage and fibrous tissue formed as part of the soft callus. If the latter hypothesis is correct, there should be expression of other cytokines late in the healing process to stimulate bone formation. We are currently engaged in a broader search for such genes.

In the unfractured bone samples, gene expression was undetectable in the older rats for all of the measured

genes shown in the Figures. An exception to this was osteocalcin for which there was a low basal level of gene expression in the older rats. In contrast, the unfractured bones from the young rats had clearly detectable amplimers for osteocalcin, type I collagen, and the type IA BMP receptor. This higher level of basal gene expression reflected the greater growth rate in the diaphyseal bone of the younger rats. Unfractured bone of both ages lacked detectable amplimers for type II collagen, reflecting the lack of appreciable cartilage synthesis in the diaphysis of the long bones.

There was a positive 18S rRNA signal in each sample. This is evidence that the failure to detect amplimers for both the matrix genes and the BMP-related genes at 4 and 6 weeks after fracture was related to decreased mRNA expression of these genes. The positive 18S signal would argue against technical difficulties in detecting expression in these samples. The unstable expression of housekeeping genes after fracture is not surprising. There is considerable increase in metabolic activity by the bone cells in response to fracture, and there is also a change in the cell population of the fracture callus in comparison to intact diaphyseal bone. It is not unusual to see change in the expression of housekeeping genes with large changes in cell activity [23,24,25,26].

We have considered whether instability could be impeding fracture healing in these older rats, and we cannot find evidence of it. All rats have intramedullary fixation. In addition, there has been no evidence of a hypertrophic callus caused by instability in the femora of the older rats that fail to reach union. Instead, after the fracture is induced, there is a slowing in the formation of the soft callus as revealed by the later peak in type II collagen. There is a more profound slowing of the bony reaction on the periosteal surface of the femoral diaphysis. The young rats have a visible periosteal reaction at week 1, while the older rats do not form mineralized tissue on the periosteal surface until the fourth week after fracture. Even if instability were present, this would not impede cytokine formation since, in a mouse model, unstable tibial fractures were associated with prolonged expression of Indian hedgehog and BMP-6 [27].

These findings have led us to hypothesize inadequate cytokine signaling in the older rats to explain the delayed fracture healing. The number of animals used in the present study, while adequate to test for early decreased gene expression in the older rats, was inadequate to compare each individual time point between the two ages. This is currently being tested in a larger experiment.

Conclusions

In summary, mid-diaphyseal fractures of the femur in rats were followed by increased mRNA expression for osteocalcin, type I collagen α1, type II collagen, BMP-2, BMP-4, and the type IA BMP receptor. In the younger rats, there was elevated gene expression until fracture healing occurred. In contrast, in the older rats, fracture healing was slower, so that cytokine gene expression returned to baseline prior to radiographic or biomechanical healing of the fractures. This decreased expression of bone-inductive genes prior to an adequate biological response suggests the absence of negative feedback regulation over fracture healing.

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Competing interests

Have you in the past five years received reimbursements, fees, funding, or salary from an organisation that may in any way gain or lose financially from the publication of this paper? No

Do you hold any stocks or shares in an organisation that may in any way gain or lose financially from the publication of this paper? No

Do you have any other financial competing interests? No

Are there any non-financial competing interests you would like to declare in relation to this paper? No

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